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13. ABSTRACT *(Maximum 200 words)*

We studied developmental changes in glucose transporter (GT) targeting in mammary gland, a prerequisite for the understanding GT targeting in breast cancer. Previously, we established that GLUT1 is targeted to Golgi during lactation. To understand the regulation of this process, we carried out subcellular fractionation and density gradient centrifugation, Western blotting, and immunofluorescence in mammary glands of mothers whose pups were prematurely weaned. We conclude: 1) There is enrichment of Golgi by GLUT1 during lactation. This is lost by 5h of weaning. 2) Enrichment can be restored by returning the pups to the mother for 5h. 3) At 10h, total cellular content of GLUT1 begins to decrease. 4) A 72 kD protein recognized by the GLUT1 antibody showed even more striking Golgi enrichment than GLUT1. 5) Intermediate MW forms at 50 and 65 kD were also observed. These each demonstrate specific patterns of appearance, disappearance, and subcellular localization, and can be deglycosylated to give aglycoGLUT1. 6) The 72 kD protein was resistant to deglycosylation. Based on the kinetics of its appearance and disappearance, its physicochemical properties which suggest it is not a GT, and based on its subcellular localization in the Golgi, p72 is an excellent candidate for a protein involved in sequestering GTs within the Golgi. 7) Ubiquitin appears to play an important role in the rapid degradation of GLUT1 and p72 during premature weaning. Identification of the cellular proteins, perhaps including p72 and ubiquitin, that constitute the mechanism by which these changes in GT targeting and amount, respectively, are achieved is a necessary step if we are to take advantage of this mechanism to alter GT targeting in cancer cells.

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Peter M Haney MD PhD 10/7/97

PI - Signature Date

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Introduction-

Prologue-

As of July 1, 1997, I became an Assistant Professor of Pediatrics at the Baylor College of Medicine in Houston Texas. I am affiliated with the Section of Neonatology and with Texas Children's Hospital and my lab is in the U.S.D.A./A.R.S. Children's Nutrition Research Center.

This move reflects my commitment to studying mammary gland biology and breast cancer. It has resulted in a major increase in the resources, personnel, and collaborators and consultants available to me. The CNRC offers excellent facilities and 35 other investigators pursuing aspects of maternal and child nutrition, including lactation and mammary gland biology, as well as epithelial cell biology. The Department of Cell Biology at the Baylor College of Medicine is open and welcoming, has a major research interest in the mammary gland, and is a major site of DOD-sponsored breast cancer research. The M.D. Anderson Cancer Center, another major site of breast cancer research, is also nearby in the Texas Medical Center. The clinical facilities of the Baylor College of Medicine offer large volumes of clinical material. The core facilities at Baylor are outstanding and are facilitating several aspects of the project. In addition to the technician which this grant funds, I now have a second technician as well. One of the first year neonatology fellows has chosen to spend two years doing research in my lab. The institutional support at Baylor is superior to what I enjoyed at Washington University. The net effect of this move on my productivity should be extremely positive.

Of course, the move from St. Louis to Houston resulted in discontinuity in the research effort, as work needed to wind down in St. Louis, and new technicians needed training in Houston. Nevertheless, I am reporting progress on studies of the weaning period. These studies show remarkably rapid and reversible changes in glucose transporter targeting. Taken together with work that I reported in my Career Development Award Annual Report last year, and include as "background of the previous work" in this report, we are well along with the first task of the Statement of Work, describing developmental changes in glucose transport in mammary gland. I anticipate no difficulty in concluding this within five more months, within the parameters of the S.O.W. Note that the second proposed task of my S.O.W., the identification of novel proteins involved in glucose transport, was not funded by my New Investigator Award, and therefore is not reported on. However, since this was funded by my DOD Career Development Award, much effort was expended during the past year in pursuing several apparently novel proteins. We are now embarking on the third task of the S.O.W., the study of glucose transporters in cancer cells, but I am not presenting any data regarding that task in this report.

Note that, since DOD policy did not allow the grant to be transferred to Baylor College of Medicine, F. Sessions Cole, M.D. graciously agreed to serve as Principal Investigator and to subcontract the work to me at Baylor College of Medicine. Therefore, I am technically submitting this report on his behalf.

a. the subject of the research

Glucose is critical to mammary epithelial cells not only because it serves as a fuel and as a building block for glycoproteins and glycolipids, but also because of its role as the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production. Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein alpha-lactalbumin(Strous, 1986). Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescient state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young.

Breast cancer cells also exhibit an increased demand for glucose, as reviewed below. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal.

b. the background of previous work

There are two mechanisms for glucose transport into cells. For most cells, the sole mechanism is the passive diffusion of glucose into cells, facilitated by the five isoforms of the glucose transporter family(Burant, et al., 1992, Mueckler, 1994). These are designated GLUT1-GLUT4, and GLUT7, in the order in which they were cloned. (GLUT5 is actually a fructose transporter(Burant, et al., 1992), and GLUT6 is a pseudogene related to GLUT5(Kayano, et al., 1990)). These isoforms exhibit distinct regulatory properties, tissue distributions, and kinetics. However, they are all integral membrane proteins containing approximately 500 amino acids. Hydropathy plots based on amino acid sequences predicted from cDNA sequence suggest that all share a common topology, which includes cytoplasmic N- and C-termini, twelve membrane spanning domains, one exofacial loop which contains an N-linked glycosylation site, and one approximately 65 amino acid intracellular loop midway through the transporter. There is a striking degree of homology among these isoforms, which are 50-65% identical in their amino acid sequence. GLUT1 is also known as the endogenous glucose transporter because of its nearly ubiquitous tissue distribution. It is important in basal glucose uptake and is usually found primarily in the plasma membrane. It is the only glucose transporter isoform convincingly shown to be expressed in mammary

epithelia. In the specialized setting of intestinal(Hwang, et al., 1991) and renal(Miller, et al., 1992, Pajor, 1994) epithelia, and possibly in pulmonary epithelia(Kemp and Boyd, 1992), glucose is also taken up by active transport across specialized membrane domains by tissue-specific isoforms of the sodium, glucose co-transporter(Hediger, et al., 1987). A possible role of this protein in mammary gland glucose transport during lactation has recently been suggested(Shennan and Beechey, 1995).

Mammary gland is unique in its requirement for free glucose within the Golgi, the site of lactose synthesis from glucose and UDP-galactose. The substrates for glycosylation of proteins within the Golgi, which occurs in many cell types, are nucleotide sugars, not free sugars. Wilde and Kuhn measured glucose uptake into rat mammary acini at different glucose concentrations, and directly measured intracellular glucose concentration, to conclude that glucose transport is rate-limiting for lactose synthesis(Wilde and Kuhn, 1981). Madon et al. measured cytochalsin B binding of fractionated rat mammary gland and found that the GLUT1 glucose transporter accounted for only about one-half of the cytochalasin B binding sites of Golgi, strongly suggesting that a unique transporter resides in the Golgi of lactating mammary gland(Madon, et al., 1990). This proposal aims to extend our understanding of glucose transport within lactating mammary gland by identifying and characterizing the molecular species responsible for glucose transport within the mammary epithelial cell and by exploring their developmental and hormonal regulation. This forms a prerequisite for understanding glucose transport in breast cancer.

Seven decades ago, Warburg(Warburg, 1923) appreciated that tumor cells show high rates of glucose uptake, glucose metabolism, and respiration(Hatanaka, 1974, Merrall, et al., 1993). Several lines of evidence suggest the value of a comprehensive understanding of glucose transport in mammary gland in the context of breast cancer. Brown et al.(Brown and Wahl, 1993) showed that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. Several groups have recently shown that the glucose analog 18-F-fluoro-2-deoxyglucose can be used to detect and stage breast cancer(Wahl, et al., 1991, Tse, et al., 1992, Nieweg, et al., 1993, Adler, et al., 1993, Crowe, et al., 1994), suggesting that an abnormally high uptake of glucose is a consistent finding in breast cancer. The MCF-7 line of breast cancer cells was established over twenty years ago(Soule, et al., 1973) and has been characterized in literally hundreds of studies since then. Inhibition of glycolysis in MCF-7 breast cancer cells by extracellular AMP markedly inhibited cell proliferation(Hugo, et al., 1992). Elegant NMR studies have recently shown that, in MCF-7 human breast cancer cells, tamoxifen inhibits glucose consumption and lactate production by 50%, compared to estrogen-treated cells, and that estrogen rescue of tamoxifen treated cells was associated with a rapid increase in glucose consumption(Furman, et al., 1992, Neeman and Degani, 1989). Glucose-6-phosphate dehydrogenase, a key enzyme of glucose metabolism, is strikingly elevated in mammary epithelial cells from patients with breast cancer compared to those with benign breast disease(Barron, et al., 1991). Its activity was also significantly increased in

morphologically normal tissue from cancer-containing breasts when compared to breasts with no known cancer, suggesting the possibility that metabolic abnormalities precede morphological changes in breast carcinogenesis(McDermott, et al., 1990). GLUT1 is the major glucose transporter isoform expressed in mammary epithelial cells. Importantly, GLUT1 is also the only known glucose transporter isoform whose gene is activated at the level of transcription in cells transformed by oncogenes such as *fps* , *src* , and *ras* (Birnbaum, et al., 1987, Flier, et al., 1987). Since this response is direct, the GLUT1 gene is an immediate early gene. Of special interest with respect to breast cancer is that the *neu* oncogene induces synthesis of GLUT1 mRNA and increases glucose uptake three-fold in fibroblasts(Sistonen, et al., 1989). Several groups have reported that between 18.8% and 67% of patients with breast cancer have elevated serum *neu* protein levels or amplification of HER-2/*neu*.(Kath, et al., 1993, Charpin, et al., 1993, Descotes, et al., 1993, Bacus, et al., 1992). Induction of GLUT1 mRNA synthesis also occurs in cells after addition of serum, peptide growth factors, and agents which increase intracellular cAMP concentration(Hiraki, et al., 1989). The two enhancer elements responsible for the responsiveness of the GLUT1 gene to growth factors and oncogenes have been characterized(Muramiki, et al., 1992).

What might the biochemistry and molecular cell biology of glucose transport in lactating mammary gland teach us about breast cancer? One fundamental answer is that, to understand the abnormal state of a cell, one must first understand its normal function and development. A more concrete rationale is suggested by the observation that high rates of glucose uptake and high levels of GLUT1 characterize breast tumors, as noted above. Furthermore, certain proteins important in lactation are also expressed in neoplastic breast tissue but not in normal, quiescent, breast tissue. Serum human alpha-lactalbumin, the mammary-specific protein cofactor that combines with galactosyltransferase to form the complex lactose synthetase, has been proposed as a marker for breast cancer(Thean and Toh, 1990). Similarly, a milk fat globule protein is highly expressed in human breast tumors(Hilkens, et al., 1986, Larocca, et al., 1991). There is also a higher molecular weight glycoprotein detectable in milk and breast carcinomas(Sekine, et al., 1985). None of these are expressed in non-lactating, non-neoplastic mammary gland.

c. the purpose and scope of the present work

In contrast to the transfer of lactose into milk across apical membrane, which occurs by vesicle fusion and is not carrier-mediated, glucose must be transported across at least three distinct cellular membranes of lactating mammary epithelial cells. Specifically, glucose must be transported from the blood across the basal plasma membrane to the mammary gland cytoplasm, from the cytoplasm across the Golgi membrane to the Golgi, where lactose synthesis occurs(Kuhn and White, 1975), and from the cytoplasm across the apical membrane to milk. No known isoforms of the glucose transporter family are known to reside primarily in the Golgi. The regulation of glucose transport

must take into account the difference in requirements of the quiescent and the lactating gland. Therefore, the specific hypotheses to be tested are:

1. Glucose transport into mammary epithelial cells is subject to a high degree of regulation.

GLUT1 and any other transporter isoforms or other novel proteins identified by pursuing the first specific aim are likely to exhibit developmental and hormonal regulation. The activities of key enzymes of lactation, such as acetyl-CoA-carboxylase, fatty acid synthetase, galactosyltransferase, phosphofructokinase, and isocitrate dehydrogenase, among others, expressed per mg DNA, rise several-fold as lactation commences(Wilde, et al., 1986). It is reasonable to expect coordinate regulation of glucose transporters. Given the crucial importance of successful lactation to continued survival of the species, and given the limited evidence that already exists, I expected to confirm that glucose transport into lactating mammary gland is, in fact, exquisitely regulated, and proposed to establish the molecular mechanisms of that regulation.

2. Abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth.

Only by comprehensively characterizing glucose transport in normal quiescent and lactating mammary gland will we obtain the data needed to understand the significance of a possible role for altered glucose transport in breast cancer. As noted above, there is substantial evidence linking glucose uptake and metabolism with cancer. I anticipate that the association between glucose metabolism and breast cancer will be confirmed. Using techniques of molecular cell biology, I will be able to test whether specific perturbations of glucose transport, including those which might be found to be associated with cancer, can of themselves confer an abnormal phenotype. I also intend to alter glucose transporter targeting in tumor cells to reduce the amount at the cell surface available for glucose uptake; this will test the hypothesis that abnormally high glucose transport is important to support tumor cell growth. There are currently no data upon which to base a prediction of the results of this experiment, although there is the precedent that the *v-sis* oncoprotein loses transforming activity when targeted to the early Golgi complex(Hart, et al., 1994). Successful reduction of glucose transport into tumor cells might offer new therapeutic possibilities.

In order to test these hypotheses, the following specific aims were chosen:

1. Description of the developmental and hormonal regulation of glucose transport in mammary gland.

GLUT1 has been identified in total plasma membrane and Golgi fractions of lactating rat mammary gland. As described above, a single method, subcellular fractionation by differential centrifugation, was used to examine subcellular

distribution, and the possibility of contamination with other cellular compartments can not be excluded. I proposed to use several methods to determine the distribution of GLUT1 in lactating rat mammary gland, in primary epithelial cells of this gland in culture(Barcellos-Hoff, 1989), and in CIT_3 and Comma 1-D cells, established mouse mammary epithelial cell lines(Danielson, et al., 1984). **Note that I am presenting data from mouse rather than rat. I have chosen to focus on mouse for several reasons. First, the established cell lines are murine. Secondly, the mammary gland transplantation technique I will soon be applying has been used in mice but not rats. Thirdly, normal mouse data would be needed to interpret data from any transgenic or knockout mice I might generate in work beyond the scope of this porposal.** Dr. Peggy Neville of the University of Colorado School of Medicine has provided me with CIT_3 cells, which she has selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions, and exhibit polarized transport (personal communication). Mice will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. Details of certain procedures proposed below, namely cell culture, [^{3}H]-2-deoxyglucose uptake assay, transfection, Western blot analysis, confocal immunofluorescence microscopy, iodixanol density gradient analysis, and immunogold labeling and electron microscopy, are found in my previous publications(Haney, et al., 1991, Haney, et al., 1995).

The subcellular distribution of GLUT1 will be examined in polarized cells. Polarity will be established by growing cells on tissue culture inserts(Parry, et al., 1987). To test the possible role of extracellular matrix in mammary epithelial glucose transport, these inserts can be coated with basement membrane matrices such as those derived from EHS tumors, which have been shown to influence gene expression and differentiated functions(Aggeler, et al., 1991, Lin, et al., 1995). The initial approach to subcellular distribution will be using confocal immunofluorescent microscopy, which results in sensitive and specific staining of GLUT1 protein. Cells will be exposed to specific affinity-purified antibodies, and then to appropriate fluorescent secondary antibodies. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining is minimized, then ruled out using appropriate controls. In this way, it is possible to localize a specific protein to basolateral membrane, apical membrane, Golgi membrane, or another intracellular compartment. An important limitation of this method is that it does not yield quantitative data. Basolateral membrane and apical membrane will be distinguishable(Sjaastad, et al., 1993) because of the polarity of cells grown on filters and because of the nature of confocal microscopy, which visualizes only one section of a cell at a time. Apical or basal membrane markers will be visualized with a second fluorescent antibody. Golgi can be stained using antibodies specific for the Golgi markers alpha-mannosidases I and II(Velasco, et al., 1993, Antony, et al., 1992). Brefeldin A is a fungal metabolite that causes disassembly of Golgi(Klausner, et al., 1992); failure of GLUT1 to alter its subcellular distribution after treatment with Brefeldin A would suggest it was not a Golgi resident(Berger, et al., 1993). Confocal immunofluorescent microscopy will also be applied to sections from lactating and non-

lactating mouse mammary gland to determine whether the subcellular localization of GLUT1 changes with differentiation.

Immunogold labeling and electron microscopy have been useful tools in defining the intracellular compartments in which glucose transporters are sequestered and in quantitating their subcellular distribution(Haney, et al., 1995, Slot, et al., 1991, Slot, et al., 1991). The distribution of endogenous GLUT1 under different conditions, such as quiescence, lactation, and neoplasia, and the distribution of heterologous transporters or other proteins of interest in experiments described below, will be studied using this method.

Subcellular distribution will be confirmed by subcellular fractionation using iodixanol density gradient centrifugation. The distibution of GLUT1 across the gradient will be directly compared with the distribution of the plasma membrane marker, 5'-nucleotidase, and the Golgi marker, alpha-mannosidase I. Protocols exist for separation of apical and basolateral plasma membrane fractions in other polarized epithelial cells(Mircheff, 1989). I intend to adapt these to mammary epithelium as well.

The possibility that other glucose transporter isoforms might be found in mammary gland has not been rigorously excluded. The simplest way to do so is to attempt to detect the mRNA for the other known transporters, GLUT2, GLUT3, GLUT4, GLUT7, and the sodium, glucose cotransporter, by Northern blotting rat mammary gland poly(A)$^+$ RNA with cDNA for each transporter. As described above, mice will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. Similar studies will be performed using neoplastic human breast tissue, and established cell lines representing both non-neoplastic (CIT_3) and neoplastic (MCF-7) states. Results will be confirmed by performing Western blots. The expression of these isoforms will also be studied in cells isolated from human milk(Lindquist, et al., 1994), and in cells isolated from reduction mammoplasty specimens. Dr. Mike Mueckler of the Washington University School of Medicine has supplied me with cDNAs for all known isoforms. If another isoform is detected, its abundance, subcellular distribution, and activity will be studied as described above for GLUT1. Dr. Mueckler has also supplied me with the available specific antibodies for all isoforms.

Established cell lines as well as cells in primary culture will be exposed to factors known to influence mammary epithelial cells, such as prolactin, growth hormone, and insulin-like growth factors(Peters and Rillema, 1992, Flint, et al., 1992, Barber, et al., 1992), among others, to determine whether there is any direct effect on glucose transport. The amounts of glucose transporters can be increased by DNA-mediated transfection of expression vectors which result in their synthesis at high levels; an increase in lactose synthesis in cells expressing supraphysiologic levels of a specific glucose transporter would confirm that glucose transport limits lactose synthesis and milk production.

2. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype.

Glucose transport in MCF-7 breast cancer cells, which exhibit polarized expression of membrane glycoproteins(Zou, et al., 1989), will be characterized by the methods described above. This will include comparisons of hormonal responsiveness of glucose transport. This observational study will describe differences between two established cell lines, the CIT_3 and MCF-7 lines, but differential expression of transporters or other regulators between the two lines, while suggestive, cannot prove the importance of a given protein.

Brown et al.(Brown and Wahl, 1993) examined twelve breast tumors and showed by immunohistochemistry that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. However, these investigators did not quantitatively assess glucose transporter targeting. I intend to carry out these studies quantitatively, at the level of mRNA as well as protein, in order to understand the magnitude of the changes in glucose transport seen in neoplasia. Tom Wheeler, M.D., of the Department of Pathology at Baylor College of Medicine, is also collaborating with me in this aspect of the study; the up to 20 breast cancer specimens per year referred to above will also be studied to determine patterns of glucose transporter expression in breast cancer. I anticipate studying at least 20 specimens before drawing conclusions.

The observational approach outlined in the previous paragraph can not distinguish whether changes in glucose transporter expression, which are, after all, likely to be observed, are central and necessary phenomena, or simply epiphenomena. The relationship between expression of a specific glucose transporter and the neoplastic phenotype will therefore be directly tested by stable transfection as described above. Normal mammary epithelial cells will be stably transfected with expression vectors containing the non-inducible CMV promoter, and will express the heterologous transporter constitutively. This will directly test the link between the transporter in question and changes in phenotype, including altered transport properties, glucose utilization, synthesis of milk components, growth characteristics, and cellular morphology. Overexpression in neoplastic cells of factors which might alter glucose transporter activity will directly test the importance of elevated glucose transport activity in contributing to tumor growth. Glucose transporter levels can also be reduced up to 80% using antisense RNA methods(Valera, et al., 1994), providing another avenue for determining the significance of GLUT1 overexpression for the neoplastic mammary cell phenotype. An expression vector based on the mouse mammary tumor virus promoter, which is active in mammary gland(Gunsburg and Salmons, 1986), confers highly inducible synthesis of heterologous proteins in epithelial cells(Hirt, et al., 1992). This will be useful in ruling out any effects of constitutive expression on membrane trafficking or differentiation, and in establishing more firmly the link between expression of heterologous protein and changes in phenotype.

An elegant method for reconstitution of mouse mammary gland from mammary epithelial cells(DeOme, et al., 1959, Medina, 1973) has been adapted to cells genetically altered in vitro(Edwards, et al., 1992) in order to study physiological and morphological correlates of oncogene expression. Normal mammary epithelial cells are isolated from one mouse and briefly put into primary culture, where a gene is introduced by retroviral infection; the cells are then transplanted into the mammary fat pad of a mouse from which the endogenous epithelium has been removed. The transplanted cells grow to reconstitute a "tree" of glandular epithelium. Transplants carrying the Wnt-1 oncogene grew in a hyperplastic pattern, showing abundant fine side-branches, but without development of alveoli. The same authors also showed that expression of the neu/erbB-2 oncogene induced epithelial abnormalities similar to human atypical hyperplasia and sclerosing adenosis(Bradbury, et al., 1993). This will be a useful method to study specifically in vivo the consequences on cellular organization and function of the overexpression of transporters or other unique factors identified in the course of this study. I will also explore the possible use of this system to understand the impact of these genetic alterations on lactose synthesis in vivo. The results of experiments with reconstituted mammary glands will serve to validate, or to question the significance of, findings from established cell lines or tumors.

Body

Experimental methods and procedures

Subcellular fractionation- (Haney, et al., 1991)- Glands were removed and homogenized in PBS (5 cc/g) with 1 mM EDTA as described above. Centrifugation for 10 min at 3000 g produced a pellet, which was resuspended and centrifuged again at 3000 g. This 3000 g pellet is the nuclear pellet. The combined supernatants of the 3000 g centrifugations were centrifuged for 10 min at 17000 g. This 17000 g pellet is the light mitochondrial pellet and is enriched in Golgi. This pellet was subjected to a self-generating iodixanol density gradient (10%-37%) by combining with a 15% iodixanol solution and centrifuging for 3 h at 180000 g. Fractions were collected using a Labconco Auto-Densiflow collector. Aliquots containing 20 µg of protein were subjected to Western blotting as described above.

Western blots- (Haney, et al., 1991; Haney and Mueckler, 1994)- Homogenates were prepared as described above and solubilized in 1%SDS. Samples were subjected to SDS-polyacrylamide gel electrophoresis, with 20 µg protein per lane. Samples were not be boiled before loading, since this distorts electrophoresis of membrane proteins. Purified human erythrocyte GLUT1 supplied by Dr. Mueckler served as standard. Proteins were transferred to nitrocellulose. Membranes were treated with Blotto for 30 min and exposed to primary antibody for 1 h. Primary antibody was a 1:1000 dilution of the highly specific, well-characterized F350, directed against the final 16 amino acids of the C-terminal cytoplasmic tail of GLUT1. Blots were washed three times for ten minutes each with PBS containing 1%SDS. Secondary antibody was horseradish peroxidase-antirabbit IgG, and signal was developed by the Amersham ECL protocol. Relative protein levels were determined by densitometry. Results shown are representative of two to three independent studies per timepoint.

Immunofluorescent microscopy- (Haney, et al., 1991; Haney and Mueckler, 1994)- Frozen tissue sections were prepared. This is the gentlest method available and preserves cell structure and antigens. A 0.5 cm x 0.5 cm x0.5 cm piece of gland was dissected and frozen gradually in Lipshaw Number 1 solution, and stored at -70°C. Using a cryostat, 5-10 µm sections were prepared. Sections were air-dried, dipped in paraformaldehyde for 2 min, washed in PBS, and placed in 1%NP40, PBS, for 5 min, then rinsed several times in PBS. Sections were exposed to peptide-affinity purified GLUT1 antibody at a concentration of up to 5 µg/ml overnight at 4°C in a humidified chamber. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining was minimized, then ruled out using appropriate controls, include antibody preabsorbed with the antigenic peptide. Sections were washed three times for 5 min each in PBS with 1% Triton X-100. Secondary antibody was FITC-labeled anti-rabbit IgG $F(ab)_2$ in PBS, 0.1% horse serum, applied at the recommended concentrations (Organon Teknika) for 1 h at room temperature.

Sections were rinsed three times for 5 min each in PBS with 1% Triton X-100. One drop of Vectashield anti-photobleaching agent was applied, then coverslips were placed. Specimens were viewed using a Olympus ix70 microscope equipped for fluorescence. Localization of signal to basolateral membrane, apical membrane, Golgi membrane, and/or other intracellular compartments was assigned by surveys of low- and high-power fields.

Assumptions

Major assumptions include specificity of the antibody, the reliability of marker enzymes in the indication of organelle distribution, and the attribution of signal on immunofluorescent cell staining to proteins of different molecular weight which are recognized by the GLUT1 antibody. The first of these has been assured by careful analysis of this antibody over several years and by the use of peptide-affinity purified antibody to reproduce the results, and by the use of appropriate controls. The second has been somewhat confusing, and we are still studying the distribution of different marker enzymes in the gradient. We do use the refractive index of individual gradient fractions to characterize them, and identify as Golgi those fractions with the characteristically low density (1.06-1.09 g/cm3) physical property of Golgi. The final concern is more a caveat than an assumption, since we know from our Western blots that the antibody specifically recognizes proteins of MW 50-80 kD, all of which would generate an equivalent signal on a per molecule basis when viewed by immunofluorescence.

Results and discussion

Since I also received a Career Development Award for this same project from the DOD, work began on the first task of the S.O.W. prior to the start of funding on this grant. (The New Investigator Award application was resubmitted during the first year of the Career Development Award.) To summarize that work, double-label immunofluorescence and subcellular fractionation by density gradient centrifugation were used to demonstrate that GLUT1 is localized, both in vitro and in vivo, in the Golgi in response to the hormonal milieu of lactation. Northern and Western blots for GLUT1 and GLUT5 indicated that the developmental regulation of glucose transporters was isoform-specific, and a rapid decline in GLUT1 levels at weaning was linked to changes in the translational efficiency or increased GLUT1 degradation.

Therefore, during the reporting period, I focused on the weaning period, since it offers the opportunity to examine whether transporter targeting is altered at a point where it is imperative that milk production be rapidly curtailed. Failure to observe changes in glucose transporter targeting would suggest that this is not an important component of the mechanism which regulates milk production. We have also begun to assess the virgin and late pregnant mouse mammary gland.

To examine the regulation of GLUT1 targeting during lactation, mouse pups were prematurely weaned at 18 days of age, the peak of milk production. This

tests the hypotheses that GLUT1 is targeted to Golgi during lactation, and that its regulation during weaning would be consistent with an important role in the regulation of lactose synthesis. Subcellular fractionation and density gradient centrifugation were employed, as described in IV.A.4.c, to isolate a Golgi-enriched fraction of mammary gland. Enrichment in Golgi but not plasma membrane was verified using galactosyl transferase as a Golgi marker and 5'-nucleotidase as a plasma membrane marker (data not shown). GLUT1 was quantitated by Western blot analysis. Experiments were done in duplicate or triplicate, and representative results are shown. As shown in **Figure 1**, *GLUT1, at 43 kD, is 5-fold enriched in the Golgi fraction of lactating mammary gland* compared to total cellular homogenate. Enrichment continued after 3 h of weaning, but *was lost by 5 h of weaning*, and diminished further thereafter. Enrichment could be restored by returning the pups to the mother for 5 h , and moreso after 15 h. A second effect of weaning, seen after 10 h, is that total cellular content of GLUT1 begins to decrease. **Figure 2** shows the *decline in total cellular and Golgi GLUT1 that occurs during natural weaning between 18 and 29 postnatal days*. Taken together, the data confirm the validity of our previous observations in mammary epithelial cells in culture and suggest that GLUT1 may be an important regulator of lactose synthesis and milk secretion.

Unexpectedly, the GLUT1 antibody also identified higher MW proteins. *A 72 kD protein showed even more striking Golgi enrichment than GLUT1, and also showed loss of Golgi enrichment during weaning* (**Figures 1 and 2**). Intermediate MW forms at 50 and 65 kD were also observed. The 50 kD and 65 kD forms were enzymatically deglycosylated using endoglycosidase F to generate aglyco-GLUT1 (data not shown). As shown in an individual density gradient result from a 5 h wean, then 15 h return of pups (**Figure 3**), *the 50 kD and 65 kD forms , which represent alternatively glycosylated GLUT1, each display specific patterns of appearance, disappearance, and subcellular localization*. For example, the 65 kD form is found only in fractions 9-12. This shows that the degree of GLUT1 glycosylation is an important determinant of its subcellular targeting. Interestingly, as also shown in **Figure 4**, *the 72 kD protein was resistant to enzymatic deglycosylation*. Note that while GLUT1 itself is deglycosylated to a 38 kD form, the 72 kD protein was not affected by this treatment. Because the 72 kD protein also was degraded within 5 h of weaning, coincident with the appearance of an 80 kD protein, an anti-ubiquitin antibody was also used for immunoblotting. Ubiquitin is an 8 kD protein important in regulation of protein degradation. **Figure 5** represents the density gradient observed after 3 h of weaning; the upper panel shows immunoblotting with antibody to GLUT1, while the lower panel was immunoblotted with antibody to ubiquitin. The data show that the *80 kD protein is the major ubiquitin-containing protein present in these fractions*, and presumably represents the ubiquitin-conjugated 72 kD protein. An important control, repeating all these experiments using antibody preabsorbed with the antigenic peptide used to generate the GLUT1 antibody, gave no signal

(data not shown). This proves the specificity of the antibody for all forms identified.

In the remainder of this report, I refer to the 72 kD protein as p72. p72 is unique to Golgi of lactating mammary gland, is highly enriched in Golgi, and is rapidly and specifically degraded upon weaning. However, although it shares the GLUT1 C-terminal antigenic determinant, it is not a glycoprotein and it presumably consists of about 700 amino acids. The known facilitated diffusion glucose transporters are glycoproteins consisting of 492-527 amino acids. Therefore, p72 is a good candidate for a regulator of glucose transporter targeting, but not for a glucose transporter itself.

Studies of virgin and late-pregnant animals were carried out after the end of the reporting period and will be discussed in next year's report.

We have also performed immunostaining for GLUT1 in virgin, late-pregnant, lactating, and weaning mice to demonstrate specific targeting of GLUT1 by an independent method. The images have not been captured due to technical issues related to computer compatibility as a consequence of my moving from St. Louis; this problem will be overcome shortly. I can report that inspection showed strong basolateral staining of GLUT1 in lactating mice, but much weaker staining in virgin mice. The intracellular staining was less than expected, and we are preparing to check other markers to see if this simply reflects inadequate permeabilization of the cell to provide antibody access to antigen, or whether there is indeed a discrepancy between the methods of immunohistochemistry and density gradient centrifugation. Interestingly, there is also very little staining for GLUT1 in late pregnant mice, suggesting that the expression of high levels of GLUT1 must be a perinatal phenomenon, and strengthening the case for glucose transport as an important regulator of lactose synthesis and milk production. We will be working to confirm this impression with further immunocytochemistry as well as by the independent method of Western blotting, and we plan to delineate the time course of GLUT1 induction during the perinatal period in an analagous manner to what we have done during weaning.

Conclusions

1. There is enrichment of Golgi by GLUT1 during lactation. This continues after 3h of weaning, but is lost by 5h of weaning, and diminishes further thereafter.

2. Enrichment can be restored by returning the pups to the mother for 5h.

3. A second effect of weaning, seen after 10h, is that total cellular content of GLUT1 begins to decrease.

4. Unexpectedly, higher MW proteins were also identified by the GLUT1 antibody. A 72 kD protein showed even more striking Golgi enrichment than GLUT1, and also showed reversible loss of Golgi enrichment during weaning.

5. Intermediate MW forms at 50 and 65 kD were also observed. These each demonstrate specific patterns of appearance, disappearance, and subcellular localization, and can be deglycosylated to give aglyco GLUT1. The degree of GLUT1 glycosylation is an important determinant of its subcellular targeting.

6. The 72 kD protein was resistant to deglycosylation. Based on the kinetics of its appearance and disappearance, its physicochemical properties which suggest it is not a glucose transporter but may be capable of associating with GLUT1 based on sharing of the C-terminal epitope, and based on its subcellular localization in the Golgi, p72 represents an excellent candidate for a protein involved in sequestering glucose transporters within the Golgi.

7. Ubiquitin appears to play an important role in the rapid degradation of GLUT1 and p72 during premature weaning.

In mice, when nursing is interrupted for as little as five hours, the targeting of GLUT1 to Golgi is rapidly but reversibly lost. After as little as ten hours, total cellular GLUT1 content begins to decrease. These changes should result in the reduced availability of glucose within the Golgi, and therefore a reduction in the rate of lactose synthesis and milk production. We can not yet comment on the kinetics of this process in human mammary epithelial cells. However, we speculate that the targeting of GLUT1 to Golgi is an important requirement for successful human lactation. I continue to believe that the identification of the cellular proteins, perhaps including p72 and ubiquitin, that constitute the mechanism by which these changes in glucose transporter targeting and amount, respectively, are achieved is a necessary step if we are to take advantage of this mechanism to alter glucose transporter targeting in cancer cells.

Recommendations in Relation to Statement of Work

I am satisfied with the Statement of Work as currently written, with the exception that I feel for reasons outlined above that working in mice has several advantages over working in rats. We have examined the weaning period and do intend to examine the perinatal period in greater detail than outlined in the Statement of Work. I do feel that accomplishing all tasks on the Statement of Work is feasible.

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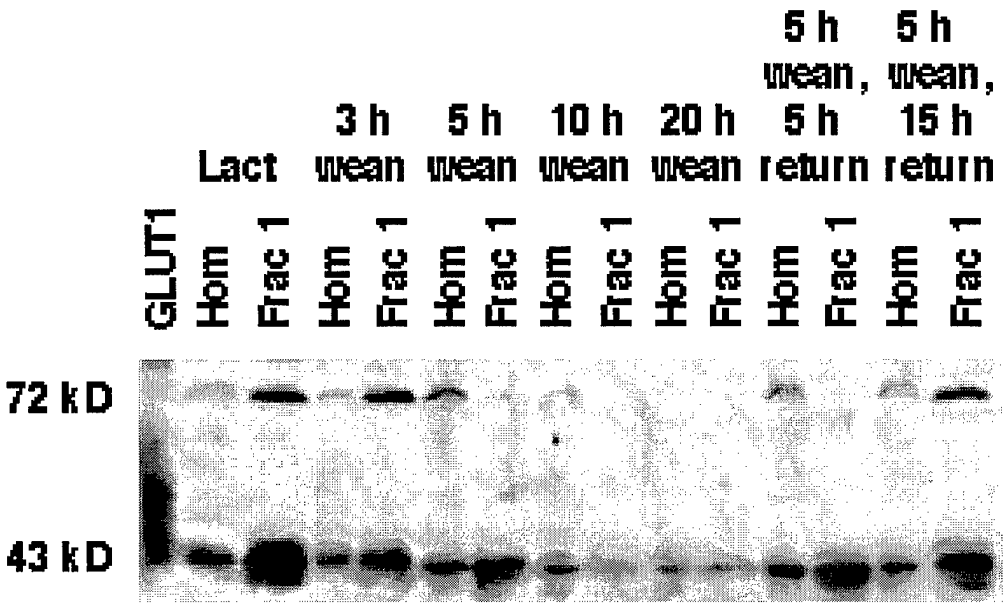

Figure 1, *GLUT1, at 43 kD, is 5-fold enriched in the Golgi fraction of lactating mammary gland* compared to total cellular homogenate. Enrichment continued after 3 h of weaning, but *was lost by 5 h of weaning,* and diminished further thereafter. Enrichment could be restored by returning the pups to the mother for 5 h. A second effect of weaning, seen after 10 h, is that total cellular content of GLUT1 begins to decrease.

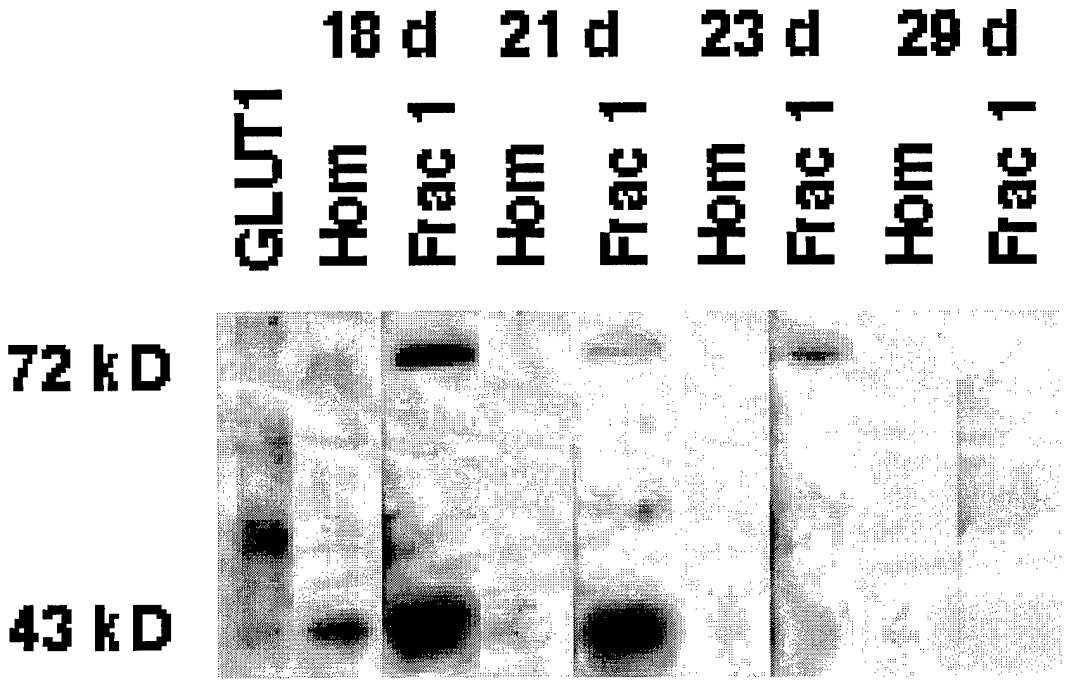

Figure 2 shows the *decline in total cellular and Golgi GLUT1 that occurs during natural weaning between 18 and 29 postnatal days.*

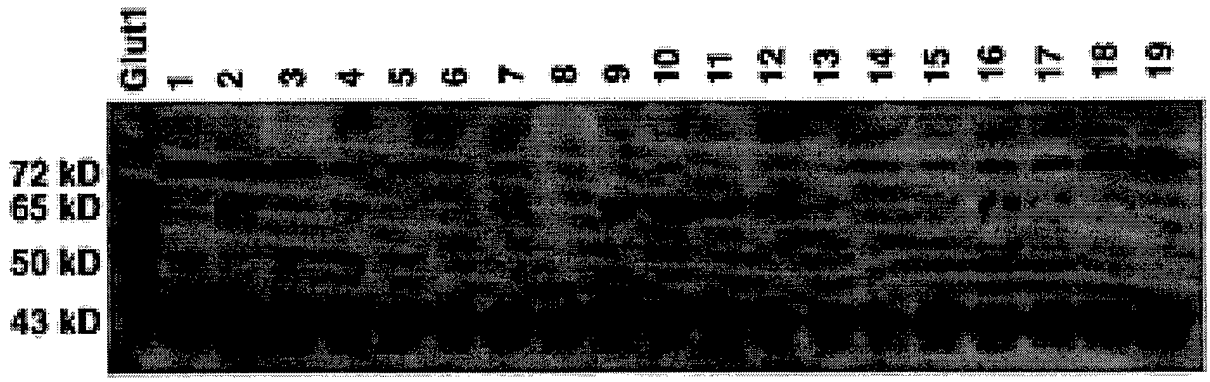

Figure 3 As shown in an individual density gradient result from a 5 h wean, then 15 h return of pups, *the 50 kD and 65 kD forms , which represent alternatively glycosylated GLUT1, each display specific patterns of appearance, disappearance, and subcellular localization*. For example, the 65 kD form is found only in fractions 9-12. This shows that the degree of GLUT1 glycosylation is an important determinant of its subcellular targeting.

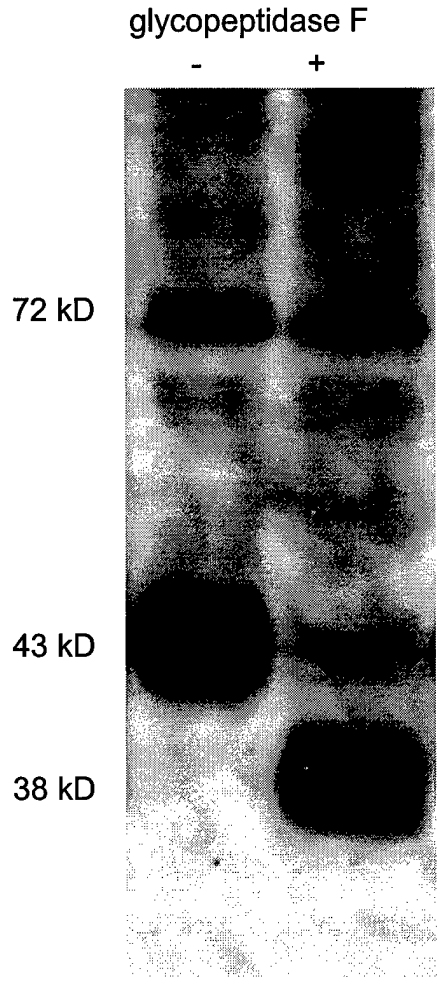

Figure 4. *The 72 kD protein was resistant to enzymatic deglycosylation.* Note that while GLUT1 itself is deglycosylated to a 38 kD form, the 72 kD protein was not affected by this treatment.

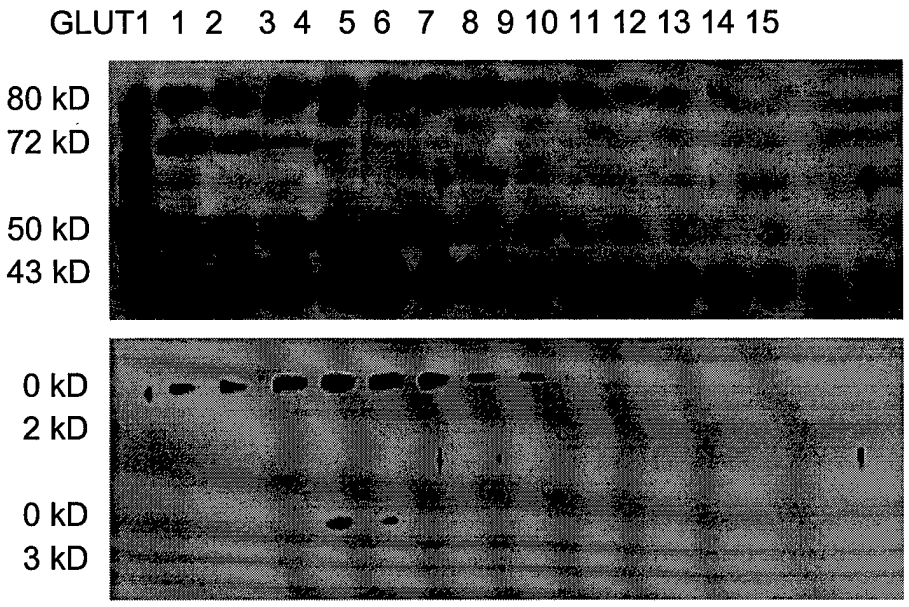

Figure 5 represents the density gradient observed after 3 h of weaning; the upper panel shows immunoblotting with antibody to GLUT1, while the lower panel was immunoblotted with antibody to ubiquitin. The data show that the *80 kD protein is the major ubiquitin-containing protein present in these fractions*, and presumably represents the ubiquitin-conjugated 72 kD protein. An important control, repeating all these experiments using antibody preabsorbed with the antigenic peptide used to generate the GLUT1 antibody, gave no signal (data not shown). This proves the specificity of the antibody for all forms identified.